PCT/GB2003/005560/

The Patent Office

PATENTS • DESIGNS • COPYRIGHT • TRADE MARKS

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed Stephen Hordley

Dated 19 January 2004

Patents Form 1/77

Patents Act 1977
(Rule 16)

The Patent Office

1/77

The Patent Office
Cardiff Road
Newport
Gwent NP9 1RH

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

1.	Your reference	44.35.79247
2.	Patent application number *(The Patent Office will fill in this part)*	0229747.1
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	Axis-Shield ASA Ulvenveien 87 N-0510 Oslo Norway
	Patents ADP number *(if you know it)*	7763873001
	If the applicant is a corporate body, give country/state of incorporation	Norway
4.	Title of the invention	Assay
5.	Name of your agent *(if you have one)*	Frank B. Dehn & Co.
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	179 Queen Victoria Street London EC4V 4EL
	Patents ADP number *(if you know it)*	166001

20 DEC 2002
23DEC02 E772738-1 D00027
P01/7700 0.00-0229747.1

		Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number			

		Number of earlier application	Date of filing *(day / month / year)*
7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application		

8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if: a) any applicant named in part 3 is not an inventor, or b) there is an inventor who is not named as an applicant, or c) any named applicant is a corporate body. See note (d))*	Yes

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	–
Description	28
Claim*(s)*	2
Abstract	–
Drawing*(s)*	–

DM

10. If you are also filing any of the following, state how many against each item.

Priority documents	–
Translations of priority documents	–
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	–
Request for preliminary examination and search *(Patents Form 9/77)*	–
Request for substantive examination *(Patents Form 10/77)*	–
Any other documents *(please specify)*	–

11. I/We request the grant of a patent on the basis of this application.

Signature [illegible] Date 20 December 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

Neil Campbell
020 7206 0600

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.*
b) *Write your answers in capital letters using black ink or you may type them.*
c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s) of the form. Any continuation sheet should be attached to this form.*
d) *If you have answered 'Yes', Patents Form 7/77 will need to be filed.*
e) *Once you have filled in the form you must remember to sign and date it.*
f) *For details of the fee and ways to pay please contact the Patent Office.*

79247.619

Assay

The present invention relates to an assay method for detecting potential for or propensity to cardiovascular disease (CVD) in a subject, e.g. a human or non-human animal, especially a mammal, and in particular to an assay method which may be used to detect a potential for CVD or a propensity to CVD before the onset of CVD symptoms noticeable by the subject.

CVD is a major source of ill health among the human population. In 1998 approximately 40 % of all deaths in the western world was a result of CVD (i.e. 1 in every 2.5 deaths). For 2002, it is estimated that, in the USA, over one million people will suffer from a new or recurrent coronary attack, and more than 40 % of the people suffering from these attacks will die. Many of these people will die suddenly without ever having been hospitalised or treated. Many will not have realised that they were susceptible to CVD.

Early or pre-emptive treatment such as a change of diet, reduction or cessation of smoking, increase in exercise, reduction of body weight, etc., has, however, a high success rate of preventing CVD or reducing the propensity to CVD. Thus if CVD or potential for or propensity to CVD can be detected, effective treatment is available.

There is accordingly a need for methods which can be used to detect CVD and especially, the potential for or propensity to CVD, before the disease has progressed beyond the stage where treatment (e.g. change of life style and/or habit) is routinely successful. In particular, there is a need for methods which can be used to detect CVD at the early stages when the symptoms are not apparent to a subject or to a third party, e.g. a physician, i.e. methods for testing "symptom-free" subjects are required.

Such methods may be used to screen the general population (i.e. in mass screening) or at-risk groups within the population, e.g. males over 40, workers in high stress jobs, individuals with unhealthy diets, individuals suffering from clinical obesity, smokers, etc. In cases where potential for CVD or propensity to CVD is diagnosed, pre-emptive treatment may be given and/or the patient may be encouraged to make adjustments to lifestyle and habit.

Likewise, where potential for CVD or propensity to CVD is detected, a patient may be submitted to further testing, e.g. using more expensive or time consuming techniques, such as ECG, with and without physical activity, radioisotope imaging of myocardial perfusion, X-ray (e.g. CT) myocardial angiography, MR myocardial angiography or perfusion imaging, etc. Thus by confirming the possible presence of, or potential for, or propensity to, CVD by using the cheap and facile assay method of the invention in an initial screen (e.g. in a mass screen of "symptom-free" healthy subjects) the likelihood of detecting or identifying undiscovered CVD, or potential for CVD, before health damage becomes irreversible is increased whilst, at the same time, unnecessary use of expensive and time-consuming tests is limited.

By "CVD" is meant any condition of the heart, arteries, or veins which disrupts the supply of oxygen to life-sustaining areas of the body such as the brain, the heart etc. Examples of CVDs are arteriosclerosis, acute myocardial infarction, angina pectoris, ischemic heart disease, cerebrovascular disease, stroke, subarachnoid haemorrhage, intra-cerebral haemorrhage, cerebral infarction, congestive heart failure, angina, heart attack and arrhythmia.

The present invention is based on the surprising finding that the protein calprotectin is a useful "marker" or "indicator" of potential for CVD or

propensity to CVD before the onset of CVD symptoms (i.e. in symptom-free subjects). In particular it has been surprisingly found that abnormally high calprotectin levels in various body fluids is indicative of susceptibility to CVD before the onset of CVD symptoms apparent to a subject or to a third party (e.g. a physician).

For the avoidance of doubt, the term calprotectin is used herein synonymously with "L1 protein", "MRP 8/14", "cystic fibrosis (associated) antigen (CFA)" and "calgranulin".

Calprotectin exists in both dimeric and trimeric forms. As a dimer, calprotectin comprises the polypeptide chains S100A8 and S100A9. As a trimer, calprotectin is a 36 kDa heterotrimeric protein with two heavy (14 kD) and one light chain (8 kD) non-covalently linked.

Calprotectin is a calcium binding protein and when bound to calcium, calprotectin is resistant to heat and to proteolysis. This allows for a wide range of assay techniques and conditions to be employed.

Epitope mapping of calprotectin shows that antibodies with specificity for the complex and/or its single protein chains may be produced. At least four separate immunogenic sites have been shown to exist on the calprotectin complex. Some antibodies recognise either the heavy or the light chain, whilst others recognise both.

Calprotectin is found in cells, tissues and fluids in all parts of the human body and is derived predominantly from neutrophils and monocytes. Calprotectin is probably present in all individuals since amongst more than 5,000 individual tested, no calprotectin free individual was found. Calprotectin is also found in rats, mice, rabbits, sheep, cattle and pigs. It is therefore an abundant ubiquitous molecule.

In vivo, calprotectin is involved in numerous

biological functions including intracellular signal transduction, neutrophil activation, inhibition of intracellular enzymes involved in cell proliferation, antimicrobial activity and in neutrophil defence. Calprotectin is also a regulatory protein in inflammatory reactions, and in this role may function to stimulate immunoglobulin production, chemotactic factor activity and neutrophil immobilising factor.

Whilst body fluid probably always comprises calprotectin, the concentration of calprotectin in various body fluids has been found to change, for example, to increase, in a number of disease conditions (e.g. inflammatory, infectious and malignant diseases). Thus measurement of the concentration of calprotectin in body fluid from patients suffering from such disease conditions (i.e. in individuals showing symptoms noticeable to the subject and/or to a third party) and comparing the calprotectin concentration determined to that in body fluid from, for example, a healthy (i.e. a non-diseased) subject may be used as a means of diagnosing such diseases.

For example, whilst the symptoms of bacterial and viral infections are very similar and diagnosis from their symptoms alone may be difficult, the concentration of calprotectin in the plasma/serum of the infected subject increases approximately 1 to 2 times with viral infections but around 1 to 18 times with bacterial infections. Thus the subject having noticed the symptoms of infection, can have the concentration of calprotectin in their body fluid measured and their infection diagnosed and treated accordingly.

Other diseases in which calprotectin may be used as a diagnostic test include: rheumatic diseases (e.g. rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus), Sjøgrens syndrome, intraocular inflammatory conditions, cystic fibrosis, acute and chronic lung disease, lung carcinoma (squamous

cells), pulmonary cancers, colorectal cancer, inflammatory bowel disease, gastric cancer, colorectal adenoma or cancer, Chrohn's disease, ulcerative colitis, gastrointestinal mucosal inflammation, urinary stones, alcoholic liver disease, oral inflammatory mucosal disease, CNS inflammatory disease (e.g. multiple sclerosis and acute encephalitis), HIV infection, secondary CNS infections in HIV infected patients, urinary tract infections, cystitis, pyelonephritis, endogenous posterior uveitis, haematological conditions (e.g. leukaemia), febrile conditions (infectious and non-infectious), acute myocardial infarction and apheresis.

The plasma concentration of calprotectin has also been found to increase during open heart surgery (Semb, A. G. et al, Eur. J. Cardio-thorac Surg. (1991) 5:363-367, Saatvedt, K. et al, Scand. J. Thor. Cardiovasc. Surg. (1996) 30: 53-60, Moen, O. et al, Perfusion (1994) 9:109-117). More specifically, Saatvedt et al. report that calprotectin concentration rises after the start of cardiopulmonary bypass and peaks 48 hours post-operatively.

It has now surprisingly been found that the potential for CVD or propensity to CVD in a subject can be assessed by determining the concentration of calprotectin in a calprotectin-containing sample taken from said subject. In other words, it has been found that determination of the concentration of calprotectin in a calprotectin-containing sample taken from a subject can be used to predict, prior to the onset of symptoms which are noticeable to the subject or to a third party (e.g. a physician) whether or how likely the subject is to suffer CVD.

By "potential for" or "propensity to" is meant the likelihood or probability that the currently symptom-free subject being tested will suffer CVD in the future. This might take the form of an index, ratio, percentage

or similar number reflective of the relative risk of CVD in the future (e.g. in the following 1-2 years, at least in the following 6 months).

Thus viewed from one aspect the invention provides an assay method for the detection of potential for CVD or propensity to CVD in a human or non-human animal subject, said method comprising assessing the concentration of calprotectin in a calprotectin-containing sample taken from said subject, e.g. a sample of blood, plasma, serum, cerebrospinal fluid, oral fluid, urine, faeces, synovial or empyema fluid.

By "assessing" it is meant that a quantitative or semi-quantitative value for the concentration of calprotectin is determined. This may be the value for the concentration of the sample as tested, e.g. after treatment to remove the cells or other sample components not being assayed for, or to concentrate or dilute the sample or to transfer the calprotectin to a separate medium, e.g. solid substrate.

Alternatively, the assessment may simply be qualitative, i.e. to indicate whether the calprotectin is above or below one or more pre-selected threshold values, e.g. values indicative of absence of potential for CVD or propensity to CVD as detectable by the assay. The precise values for these threshold values or other reference values for calprotectin may depend on the nature of the sample, the age, weight, sex and species of a subject and may be determined in a routine manner by measuring the calprotectin concentration of the relevant body fluid of equivalent subjects without CVD or with CVD at various stages of development.

A value indicative of calprotectin concentration determined or "assessed" in accordance with the method of the invention may be an absolute concentration of calprotectin or may alternatively be an index, ratio, percentage or similar number reflective of the concentration of calprotectin.

A body sample used in the assay method of the invention may be any calprotectin-containing sample, e.g. a body fluid or tissue sample, or a suspension etc. Preferably, the sample will be a body fluid, e.g. urine, cerebrospinal fluid, oral fluid, synovial fluid or empyema fluid, or more preferably, blood or a blood derived sample. When this is the case, the sample used for analysis will preferably be cell-free (e.g. serum or plasma). Alternatively faeces may be used.

The sample may be treated prior to being used in the assay method of the invention. Thus the sample may be treated to remove any cells and/or any sample components not being assayed for. The sample may also be treated to concentrate or dilute the sample or to transfer the calprotectin to a separate medium, e.g. solid substrate. For example the sample may be diluted by adding a buffer or other aqueous medium. Alternatively, a sample, particularly a plasma or serum sample, may be used directly.

The sample is optionally treated with calcium or a calcium mimic (e.g. ions of another alkaline earth metal), prior to being used in the assay method of the invention. The calcium or calcium mimic may be any form which provides Ca^{2+} ions (e.g. $CaCl_2$). If calcium is used then preferably sufficient calcium or calcium mimic is added to the sample to saturate the calcium binding sites of calprotectin. For example, a ten molar excess of calcium source may be added, more preferably, a five molar excess or especially preferably a three molar excess.

While assays for calprotectin are known and may be used in the method of the invention, there has not previously been any suggestion that calprotectin is a marker or indicator of potential for CVD or propensity to CVD. In other words, there has not previously been any suggestion that the calprotectin concentration of symptom-free subjects might be used as a marker or

indicator of potential for or propensity to CVD and, in particular, there has not been any suggestion that calprotectin concentration might be used as the marker or indicator in an assay method suitable for mass screening of healthy (i.e. symptom-free) subjects.

Any known assay method for calprotectin may be used in the assay method of the invention. Thus, for example, the method disclosed in US-A-4833074 (Fagerhol et al.) for the isolation of calprotectin and for the subsequent production of monospecific anti-sera thereto may be used to produce anti-calprotectin antibody for use in any conventional assay method. The anti-calprotectin antibodies produced may be used, for instance, in enzyme linked- and radio- immunoassays.

A NycoCard® (Axis-Shield PoC, Oslo, Norway) immunoassay format for calprotectin may also, for example, be used. This assy uses a solid phase, sandwich-format in which the test device comprises a membrane coated with immobilised anti-calprotectin antibodies. Thus the sample (optionally diluted) is applied to the device and when the sample flows through the membrane, any calprotectin present is captured. The calprotectin immobilised on the membrane is then treated with a gold-antibody conjugate which binds to the calprotectin-antibody complex and the intensity of colour (due to the gold beads), as determined by absorbance of red light, is proportional to the amount of calprotectin. The concentration of calprotectin can therefore be calculated from a calibration curve prepared in the conventional manner.

Alternatively, the commercial test for calprotectin, for example, in faeces (available from Eurospital®) may be used. This assay uses a polyclonal antibody against calprotectin in an enzyme linked immuno-sorbent assay system. Thus calprotectin present in a sample taken from a subject becomes bound to antibody, which is adsorbed to the surface of a plastic

well. A substrate for the enzyme is then added and the intensity of the coloured product produced is proportional to the amount of enzyme and therefore to the amount of calprotectin. The concentration of calprotectin can therefore be calculated from a calibration curve prepared in the conventional manner.

Indeed, both mono- and polyclonal anti-calprotectin antibodies are available commercially. Egg and rabbit polyclonals are, for example, are available from Norwegian Antibodies AS and Axis-Shield Diagnostics respectively, whilst mouse monoclonal antibody may be obtained from Dako A/S, Denmark. Any anti-calprotectin antibody obtained, for example, by any conventional technique for making antibodies, may be used in the method of the invention. For instance, rabbit anti-calprotectin antibody as well as monoclonal antibodies can be produced according to the protocol described in Harlow and Lane (1988), Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, NY.

Alternatively, anti-calprotectin antibodies may be prepared by regularly injecting a calprotectin-containing solution into chickens, and then collecting the yolks of the chicken's eggs. Chicken egg polyclonal antibody can then be isolated according to conventional techniques and purified by affinity chromatography.

Preferably, the assay method of the invention is used for mass screening of healthy (e.g. CVD symptom-free subjects). Where potential for CVD or propensity to CVD is detected, the subject may be subjected to further testing (e.g. using more expensive techniques specific to CVD) to confirm the presence or absence of CVD.

In general, besides the sample under evaluation, calibration samples with known calprotectin content will also be assessed in the performance of the assay method. Such determinations can be used to plot a calibration curve from which the calprotectin content of the sample

under investigation may be determined. The nature of the calibration samples and selection of conversion or adjustment factors used in the determination of the calprotectin may vary depending, for example, on the manner in which the calprotectin is detected in the assay technique actually used and on other aspects of the method which affect the assay result, for example, buffer composition, assay conditions etc.

Typically calibration samples having calprotectin contents of 0 to 5000 mg/L will be used. The reference range within which the value for calprotectin concentration will generally be found is 0.1 to 10 mg/L.

In general, the concentration of calprotectin in the serum and plasma of humans with little or no potential for CVD or propensity to CVD will be in the range 0.01-0.75 mg/L. More specifically, the concentration of calprotectin in the serum and plasma of such humans will be in the range 0.05-0.70 mg/L, even more specifically 0.10-0.66 mg/L. For example, the concentration of calprotectin in the serum or plasma of a female with little or no potential for CVD or propensity to CVD will be in the range 0.09-0.53 mg/L, for example, 0.31 mg/L. The concentration of calprotectin in the serum or plasma of a male with little or no potential for CVD or propensity to CVD will be in the range 0.12-0.66 mg/L, for example, 0.39 mg/L.

A calprotectin concentration in serum or plasma of greater than 0.75 mg/L will generally be strongly indicative of potential for CVD or propensity to CVD. Thus a threshold value above which the assay may be held to be predictive of CVD potential or propensity to CVD may generally be about 0.67 mg/L, more preferably about 0.70 mg/L, especially about 0.76 mg/L.

In general, the concentration of calprotectin in the faeces of humans with little or no potential for CVD or propensity to CVD will be in the range 0.01-10 mg/L. More specifically, the concentration of calprotectin in

the faeces of such humans will be in the range 0.05-9.0 mg/L, even more specifically 0.50-8.0 mg/L.

A calprotectin concentration in faeces of greater than 10 mg/L will generally be strongly indicative of potential for CVD or propensity to CVD. Thus a threshold value above which the assay may be held to be predictive of CVD potential or propensity to CVD may generally be about 9 mg/L, more preferably about 10.5 mg/L, especially about 11 mg/L.

However, the threshold values are better calculated from calprotectin determinations using the same assay techniques for the same body fluid sample type from a range of patients of similar type (age, sex, weight, species etc.) from healthy through early stage CVD to serious CVD. Even more preferably, the threshold values will be values determined for the same patient at an earlier, healthy stage. Thus, particularly at-risk, individuals could monitor their calprotectin levels on a routine basis (e.g. every 6-months to 1 year) in mass screening programmes.

In a preferred assay method of the present invention, said method further comprises additionally assessing the concentration of another marker for potential to CVD in the sample taken from the subject. Examples of suitable markers may be homocysteine, activated factor XII, cholesterol, cholesterol:HDL ratio, fibrinogen, tissue-type plasminogen activator, Factors V, VII and VIII, lipoprotein (a), von Willebrand factor antigen, plasmin-α2 antiplasmin complex, prothrombin fragment 1+2, thrombin-antithrombin III complex, fibrinopeptide A, fibrin degradation products, D-dimer, activated protein C-resistance, factor VIIc and VIIa, thrombin, serum amyloid A, vascular adhesion molecules and coronary calcium.

More preferably the assay method of the present invention further comprises additionally assessing the concentration of C-reactive protein (CRP) in the sample

taken from the subject. Preferably, the concentration of CRP is assessed simultaneously or sequentially to said calprotectin assay.

The measurement of CRP may be effected using any standard immunoassay technique (e.g. ELISA, RIA etc.) or may be determined by NycoCard® (available from Axis-Shield PoC, Oslo, Norway).

A calprotectin concentration in serum or plasma of greater than 0.75 mg/L in addition to a CRP concentration of greater than 1.75 mg/L will generally be strongly indicative of potential for CVD or propensity to CVD. Preferably, the presence of the above-mentioned concentrations is more strongly indicative of potential for CVD or propensity to CVD than a calprotectin or CRP concentration alone.

Thus threshold values of calprotectin and CRP above which the assay may be held to be highly predictive of CVD potential or propensity to CVD may generally be about 0.67 mg/L and 1.75 mg/L respectively, more preferably about 0.70 mg/L and 2.00 mg/L respectively, especially about 0.76 mg/L and 2.25 mg/L respectively.

Viewed from a further aspect, the present invention provides an assay kit for use in the method of the invention, said kit comprising reagents and instructions for the performance of the assay method and for the interpretation of the results and, optionally, calprotectin-containing reference samples, and optionally, a detector. Preferably, said assay kit further comprises the reagents and instructions for determination of CRP concentration.

The instructions in the kit may for example be in the form of a label, a manual or an instruction leaflet; however, they may instead take the form of a computer program or a data carrier, e.g. a computer disk.

The detector, where present, will generally be one capable of detecting a reporter species, e.g. a spectrometer, a nuclear radiation detector, a scattered

light detector, etc.

The reagents will be agents suitable for calprotectin determination, e.g. suitable reagents are specified in the literature associated with the available tests for calprotectin such as from Eurospital® and NycoCard (available from Axis Shield ASA, Oslo, Norway) cited herein. The reagents mentioned in US-A-4833074 may also be suitable.

A particularly preferred assay method for assessing the concentration of calprotectin in the present invention is a particle-based immunoassay. This is a sensitive technique which is based on turbidimetric determination of the calprotectin concentration. Turbidimetric determination has the advantage that no solid surface is required for physical separation in the assay and numerous washing and/or separation steps are not required. Thus it is quick and easy to perform and may, for instance, be automated.

For turbidimetric determination of calprotectin concentration, the calprotectin-containing sample will be a body fluid, e.g. urine, cerebrospinal fluid, oral fluid, synovial fluid or empyema fluid, or more preferably, blood or a blood derived sample. When this is the case, the sample used for analysis will preferably be cell-free (e.g. serum or plasma).

Thus the sample may be treated to remove any cells and/or any sample components not being assayed for. The sample may also be treated to concentrate or dilute the sample or to transfer the calprotectin to a separate medium, e.g. solid substrate. For instance, the sample may be diluted by adding water, a buffer or other aqueous medium. Alternatively, a sample, particularly a serum or plasma sample, may be used directly.

The sample is optionally treated with calcium or a calcium mimic, prior to being used in the assay method. The calcium or calcium mimic may be any form which provides Ca^{2+} ions (e.g. $CaCl_2$). If calcium is used then

preferably, sufficient calcium or calcium mimic is added to the sample to saturate the calcium binding sites of calprotectin. For example, a ten molar excess of calcium source may be added, more preferably, a five molar excess or especially preferably a three molar excess.

Opacity, for turbidimetric determination of calprotectin concentration, will generally be generated by contacting the calprotectin-containing sample, or an aliquot thereof, with an anti-calprotectin antibody, antibody fragment or mixture of anti-calprotectin antibodies (e.g. a mixture of monoclonal antibodies). The egg polyclonal anti-human calprotectin commercially available from Norwegian Antibodies AS may, for example, be used to generate opacity. Any anti-calprotectin antibody obtained, for example, by any conventional technique for making antibodies, may be used in the method of the invention.

The antibodies, or antibody fragments, which are used for turbidimetric determination of calprotectin concentration preferably show no or little cross reactions with other blood proteins that may be present in the eluate. The quantity of antibody, or antibody fragment, used should of course be optimised against calprotectin-containing standard samples as opacification arises from the hook effect whereby multiple calprotectin binding generates the opacification centres. Calprotectin, as mentioned above has numerous antibody binding sites, and is particularly suitable for detection in such an assay.

In one preferred embodiment, the anti-calprotectin antibody, or antibody fragment, may be immobilised by binding or coupling, either directly or indirectly, to any well known solid support or matrix which is commonly used for immobilisation. Preferably the solid support or matrix takes the form of particles, preferably nanoparticles, and conveniently may be made of glass,

silica, latex, metal (e.g. gold) or a polymeric material (e.g. polyethylene).

Binding or immobilisation of the anti-calprotectin antibody or antibody fragment may be achieved using any conventional technique. For example, avidin (available from Pierce Chemical Company) may be immobilised on chloromethyl activated polystyrene nanoparticles (available from Interfacial Dynamic Corporation, US) by agitation in buffer (e.g. at room temperature for 24 hours) and then used in conjunction with biotin labelled anti-calprotectin antibodies (prepared according to conventional techniques in the art). Thus, for example, plasma taken from the subject to be tested for potential for, or propensity to, CVD is added to a solution of avidin-coated nanoparticles in a quartz cuvette of a spectrophotometer, followed by the addition of biotin labelled anti-calprotectin antibody. Turbidimetric readings are then taken.

Alternatively, the biotin labelled antibodies may be added prior to the addition of plasma or serum. In other words, whilst the same reagents are typically used regardless of the instrument used for turbidity detection, the precise sequence in which the various reagents are added may vary. Generally, the sequence used should be in accordance with the instructions accompanying the spectrophotometer used (e.g. a Shimadzu UV-160 spectrophotometer).

Turbidimetric readings are made (i.e. the light absorption at a suitable wavelength is measured at regular intervals) and the light absorption relative to a reference is determined. Optionally, multiple wavelength instruments may be used to make turbidimetric readings and may provide more precise results. Suitable instruments for taking turbidimetric readings include the Cobas Mira, Roche Integra and Merck's Turbiquant.

In an alternative experimental set-up, the anti-calprotectin antibody, or antibody fragment, may be

immobilised directly on chloromethyl activated nanoparticles (available from Interfacial Dynamic Corporation, US). For instance, anti-calprotectin antibody (e.g. the egg polyclonal antibody available from Norwegian Antibodies AS) may be mixed with the above-mentioned activated particles in a buffer (10 mM borate, 15 mM sodium chloride, pH 9.0) and agitated (e.g. at room temperature for 24 hours) to furnish anti-calprotectin antibody-coated nanoparticles. Such nanoparticles may be used for turbidimetric determination of calprotectin concentration by adding them to a sample of plasma or serum, taken from the subject to be tested for potential for, or propensity to CVD, in a buffer and taking turbidimetric readings in kinetic mode.

Alternatively, the plasma or serum may be added to the anti-calprotectin antibody-coated nanoparticles. In other words, whilst the same reagents are typically used regardless of the instrument used for turbidity detection, the precise sequence in which the various reagents are added may vary. Generally, the sequence used should be in accordance with the instructions accompanying the spectrophotometer used (e.g. a Shimadzu UV-160 spectrophotometer).

Examples of automated robots which are suitable for taking turbidimetric readings in accordance with the assay method of the invention include the Cobas Mira and Hitachi 711, both of which are available from Roche Diagnostics.

The particles to which the antibody, or antibody fragment, may be bound are typically spherical with a diameter of 1-150 nm, preferably 10-90 nm and more preferably 15-60 nm, for example, 44 nm.

Alternatively, the particles preferably have a diameter which does not itself enable absorption of the wavelength of light used for spectrophotometric determination. Thus the suspension of coated

nanoparticles is approximately transparent until calprotectin induced aggregate formation occurs resulting in the formation of aggregates having a larger diameter. Such aggregates have the ability to absorb the wavelength of light used by the spectrophotometer.

Further, the particles are preferably substantially all of the same size, more specifically all of the same diameter. Preferably, monodisperse metal (e.g. gold) or polymer particles are used. Monodisperse polymer particles are available from Dynal Biotech AS, Oslo, Norway.

Whilst not wishing to be bound by theory, it may be that the use of immobilised antibody or antibody fragments increases the sensitivity of the assay by increasing the size of any calprotectin derived opacity generating sites and therefore the amount of light scattered therefrom. By using a solid support or matrix (e.g. nanoparticles) which is substantially all of the same size it may be that the sensitivity of the turbidimetry assay is further increased.

As is routine in turbidimetric assays, a polymeric opacification enhancer, such as polyethyleneglycol, is preferably also added to the eluate.

Before the turbidimetric determination is made, the fraction, antibody or antibody fragment, preferably bound to a nanoparticle, and optionally enhancer may be incubated for a short period, e.g. 5 minutes to an hour, preferably about 10 minutes, at room temperature. Optionally, in determining calprotectin concentration using the turbidimetry technique, a kinetic reading mode may be used.

The light used in the determination of opacification should have an appropriate wavelength. In this regard it was found that use of a 300-450 nm filter, preferably a 340 nm or a 405 nm filter, furnished particularly good results.

In general, in addition to the sample under

evaluation calibration samples with known calprotectin contents will also be assessed in the performance of the assay method. Such determinations can be used to plot a calibration curve from which the calprotectin content of the sample under evaluation can be determined. Preferably calibration samples having calprotectin contents of up to 5000 mg/L (e.g. 1500, 1000, 750, 250, 100) or up to 100 mg/L (e.g. 75, 50, 25, 5, 1.0 and 0.5 mg/L) will be used.

The above described turbidimetric assay for the determination of calprotectin is surprisingly reliable, quick, cheap, facile and amenable to automation. This is in contrast to the currently available assay methods which are relatively complex and are not directly applicable to the automated multi-task diagnostic machines commonly used by diagnostic laboratories.

Indeed there is a continuing need for cheap, reliable, quick and facile calprotectin assays for use in diagnostic techniques.

Thus, according to a further aspect, the present invention provides a method for the determination of calprotectin in a calprotectin-containing body fluid, said method comprising the steps of:

(a) obtaining a calprotectin-containing liquid sample of or derived from said fluid;

(b) contacting said sample of said body fluid with an, optionally nanoparticle-bound, anti-calprotectin antibody or antibody fragment, to bind said calprotectin;

(c) optionally, adding an opacity enhancer; and

(d) assessing the calprotectin content by turbidimetry.

Such as assay may be useful in the diagnosis of various disease conditions which are characterised by abnormal levels (e.g. high levels) of calprotectin. Such disease conditions include: rheumatic diseases (e.g. rheumatoid arthritis, juvenile rheumatoid

arthritis, systemic lupus erythematosus), Sjøgrens syndrome, intraocular inflammatory conditions, cystic fibrosis, acute and chronic lung disease, lung carcinoma (squamous cells), pulmonary cancers, colorectal cancer, inflammatory bowel disease, gastric cancer, colorectal adenoma or cancer, Chrohn's disease, ulcerative colitis, gastrointestinal mucosal inflammation, urinary stones, alcoholic liver disease, oral inflammatory mucosal disease, CNS inflammatory disease (e.g. multiple sclerosis and acute encephalitis), HIV infection, secondary CNS infections in HIV infected patients, urinary tract infections, cystitis, pyelonephritis, endogenous posterior uveitis, haematological conditions (e.g. leukaemia), febrile conditions (infectious and non-infectious), acute myocardial infarction and apheresis.

A body sample used in the turbidimetric assay method may be any calprotectin-containing sample, e.g. a body fluid or tissue sample, or a suspension etc. Preferably, the sample will be a body fluid, e.g. urine, cerebrospinal fluid, oral fluid, synovial fluid or empyema fluid, or more preferably, blood or a blood derived sample. When this is the case, the sample used for analysis will preferably be cell-free (e.g. serum or plasma). Alternatively faeces may be used.

Preferably, the body sample is selected to provide the most sensitive indication of the disease being diagnosed. Thus whilst blood, plasma or serum might be tested to diagnose infections (e.g. HIV, bacterial infection), rheumatic disease, leukaemia etc., faeces might be tested during diagnosis of diseases associated with the gastrointestinal tract (e.g. Crohn's disease, ulcerative colitis, colorectal cancers).

Viewed from yet a further aspect, the invention provides a kit for a diagnostic turbidimetric assay according to the invention, said kit comprising:

preferably, a calprotectin solution of known

concentration and more preferably a set of such solutions having a range of calprotectin concentrations;

one or more anti-calprotectin antibodies or antibody fragments, optionally immobilised on a solid support (e.g. nanoparticles);

preferably, a light transmitting vessel;

preferably, an opacification enhancer; and

preferably, a detector.

If desired an automated apparatus may be arranged to receive a calprotectin-containing body fluid sample, apply the anti-calprotectin antibody or antibody fragment, optionally immobilised on a solid support (e.g. nanoparticles), optionally apply an opacification enhancer, and determine calprotectin content. Such an apparatus is also deemed to fall within the scope of the invention.

The invention will now be described further with reference to the following non-limiting Examples.

Example 1: Anti-Calprotectin Antibody

(a) Isolation of Calprotectin

Calprotectin may be isolated according to the methods described in Examples 1 and 2 of US-A-4,833,074 (Fagerhol).

Calprotectin may alternatively be purified from human buffy coats. A cell-suspension in 2.5 mM EDTA is made by the addition of EDTA (50 mM, pH 7) to cells. The cells are then washed in 160 mM ammonium chloride/10 mM sodium hydrogen carbonate for 3 minutes and centrifuged (160 xg) for 10 minutes at 4°C. The resulting pellet is washed in EDTA (2.5 mM)/NaCl (150 mM) and centrifuged (55 xg) for a further 10 minutes at 4°C. The pellet is then resuspended in 0.625 mM EDTA/ 18.75 mM Diemal, pH 7.4 and frozen at -70°C for at least 24 hours.

Following thawing, the resulting material is centrifuged (at 3700 xg) for 30 minutes, then the supernatant is removed and filtered (with a 0.45 μm filter available from Millipore), then loaded onto a DEAE (diethylaminoethyl) Sepharose ion-exchange column (available from Pharmacia), pre-prepared using a binding buffer (e.g. 0.63 mM EDTA/18.75 mM Diemal, pH 7.4). Any non-binding material passes through the column and is eluted. Once all of the non-binding material is eluted from the column, pure calprotectin is eluted using a calcium-containing elution buffer (e.g. 75 mM Diemal buffer/10 mM $CaCl_2$). About 25 mg calprotectin is obtained per buffy coat.

(b) Preparation of Anti-Calprotectin Antibody

Anti-calprotectin antibodies may be prepared according to the method described in Example 3 of US-A-4,833,074 (Fagerhol).

Chicken egg polyclonals may alternatively be prepared. A solution comprising calprotectin (0.5 mg/ml) and Freund's adjuvant is injected into chickens every 14 days four times (or for two months), and then once every 1 month. After 12 weeks, the eggs of the calprotectin-injected chicken may be collected and their yolks removed (without the film). Following dilution in HCl (5 mM), the yolk is centrifuged and the supernatant is collected. The supernatant is then filtered and treated with saturated ammonium sulfate to a final concentration of 3.8 M. The mixture is centrifuged and the precipitate produced is collected and dissolved in buffer (0.11 M sodium acetate, 0.15 M NaCl, pH 7.4). The resulting solution is finally dialysed with a membrane having a pore size of 10,000 kD and then purified by affinity chromatography.

The column typically used for affinity chromatography comprises an activated matrix of

succinimide-activated sepharose (HiTrap NHS activated available from Amersham-Pharmacia) which is suitable for the immobilisation of calprotectin. More specifically, the activated resin reacts spontaneously, at pH 7-8, with free amines in the calprotectin. For chromatography the dialysis solution is usually diluted to a concentration of about 3 mg/ml in PBS prior to its application to the column. The anti-calprotectin antibodies are subsequently eluted using 6 M urea in ice cold PBS. Following elution, the anti-calprotectin antibody containing fractions are immediately diluted and dialysed in PBS.

Example 2: Turbidimetric Assay for Calprotectin

(a) Preparation of Avidin-coated Nanoparticles

600 μl of 4.2% w/v chloromethyl activated nanoparticles (diameter 44 nm) available from Interfacial Dynamic Corporation, US are dialysed against water with a membrane having a pore size of 10,000 kD 0.5 ml of a borate (10 mM) and sodium chloride (15 mM) solution at pH 9.0 is added and mixed. 10 mg avidin, dissolved in 0.5 ml of a 10 mM borate and 15 mM NaCl solution at pH 9 (available from Pierce Chemical Company) is added and the mixture is agitated at room temperature for 24 hours. 40 μl of glycin solution (2M, pH 9.0) is then added and the mixture is agitated for a further 4 hours at room temperature.

The particles are then diluted to a volume of 100 ml and diafiltrated, firstly in 500 ml of a 10 mM borate and 15 mM sodium chloride solution at pH 9.0 and secondly in a 25 mM Tris, 150 mM sodium chloride and 0.01 % Tween® 20 solution at pH 7.4 (available from Sigma US) using a Pellicon XL Filter (cut off 300,000) and a labscale TTF System (available from Millipore) in accordance with the instructions supplied from the

instruments suppliers. The desired concentration of avidin-coated nanoparticles is finally obtained by centrifugation and re-suspension of the particles in a 25 mM TRIS, 150 mM sodium chloride and 0.01% Tween® 20 solution. Any aggregates formed during this preparation procedure may be removed by slow centrifugation.

(b) Assay for Calprotectin using Avidin-coated Nanoparticles

A suspension having a concentration of about 0:30 mg particles of the above-described avidin-coated nanoparticles per ml is prepared by centrifugation and re-suspension of the above-described preparation in a 25 mM TRIS, 150 mM NaCl, 0.1% Tween® 20 and 2 % PEG 6000 solution at pH 7.4 (available from Sigma). 500 μl of this particle suspension is mixed with a plasma sample (about 20 μl), taken from a subject being tested for propensity to CVD, in a reading quartz cuvette of a recording spectrophotometer (e.g. a Shimadzu UV-160). The absorption of 340 nm monochromatic light is recorded and after 60s, 75 μg of anti-calprotectin antibody labelled with 0.15 nmol biotin (e.g. biotin labelled affinity purified egg polyclonal purchased from Norwegian Antibodies AS, Norway), diluted in 50 μl of a 25 mM TRIS, 150 mM NaCl and 0.1% Tween® 20 solution at pH 7.4 is added to the quartz cuvette and mixed. The absorption of 340 nm monochromatic light is immediately recorded using a reference cuvette containing a solution of 25 mM TRIS, 150 mM NaCl and 0.1% Tween® 20 at pH 7.4, and again at regular intervals (e.g. every 2 minutes) until about 15 minutes has elapsed. The increase in absorption at each time point is calculated in accordance with standard turbidimetric reading in kinetic mode or "end-point" readings. That is, the increase in light absorption at each time-point is calculated relative to the reading made prior to the

addition of antibody-coated nanoparticles and/or at the end of the recording.

A calibration curve is constructed by carrying out an identical procedure with standards having a known concentration of calprotectin. The concentration of calprotectin in the sample can then be calculated from the calibration curve.

Example 3: Alternative Turbidimetric Assay for Calprotectin

(a) Preparation of Anti-Calprotectin Antibody Coated Nanoparticles

1 ml of 4.2 % w/v chloromethyl activated nanoparticles (diameter 44 nm) available from Interfacial Dynamic Corporation, US are dialysed against water with a membrane having a pore size of 10,000 kD. 0.5 ml of a 10 mM borate and 15 mM sodium chloride solution at pH 9.0 is then added. Following addition of 17 mg of purified anti-calprotectin antibodies (e.g. affinity purified egg polyclonal antibodies available from Norwegian Antibodies AS, Norway) in 0.5 ml of a 10 mM borate and 15 mM sodium chloride solution at pH 9.0, the mixture is agitated for 24 hours at room temperature. 40 μl of a glycin solution (2 M at pH 9.0) is then added and the mixture is agitated for a further 4 hours at room temperature.

The particles are then diluted to total volume of 100 ml and diafiltrated, firstly in 500 ml of a 10mM borate and 15 mM sodium chloride solution at pH 9.0 and secondly, in 25 mM TRIS, 150 mM sodium chloride and 0.1% Tween® 20 solution at pH 7.4 (available from Sigma, US) using a Pellicon XL filter (cut of 300,000) and a labscale TFF system (available from Millipore) in accordance with the instructions supplied from the instruments suppliers. The desired concentration of

anti-calprotectin antibody-coated nanoparticles is finally obtained by centrifugation and re-suspension of the particles in a 25 mM TRIS, 150 mM sodium chloride and 0.1% Tween® 20 solution. Any aggregates formed during this preparation procedure may be removed by slow centrifugation.

(b) Assay for Calprotectin using Anti-calprotectin Antibody-coated Nanoparticles

A suspension comprising 400 μg of the above-described antibody-coated nanoparticles in 50 μl of a 25 mM TRIS, 150 mM NaCl and 0.1% Tween® 20 solution at pH 7.4 is prepared.

Simultaneously, 20 μl of plasma, taken from the subject being tested for potential for CVD, in 500 μl assay buffer (25 mM TRIS, 150 mM NaCl, 0.1 % Tween® 20 and 2% PEG 6000 at pH 7.4 (available from Sigma) is put in a reading quartz cuvette of a recording spectrophotometer (e.g. Shimadzu UV-160) and the light absorption of 340 nm monochromatic light is measured. After 60s, the above-mentioned suspension comprising 400 μg of antibody-coated nanoparticles is added, and mixed in the cuvette. The light absorption immediately after adding the antibody-coated nanoparticles is recorded, and again at regular intervals (e.g. every 2 minutes) until about 15 minutes has elapsed. The increase in light absorption at each time-point is calculated relative to the reading made prior to the addition of antibody-coated nanoparticles and/or at the end of the recording. In other words, turbidmetric readings in kinetic mode or "end-point" readings are made.

A calibration curve is also constructed by carrying out an identical procedure with standards having a known concentration of calprotectin. The concentration of calprotectin in the sample can then be calculated from the curve.

Example 4: Comparison of Calprotectin and other markers for detection of potential for CVD or propensity to CVD

Coronary calcification has been shown to be strongly associated with the occurrence of CVD and has also been demonstrated to be a useful method for predicting potential for CVD (e.g. mycocardial infarction or stroke).

The extent of coronary calcification is quantitatively, measured used electron-beam computed tomography (EBCT) and is represented by a calcium score (CS). A high calcium score represents a high level of calcification and a high risk of developing CVD.

In the following study the CS of 200 subjects aged 45 or greater was tested as well as their calprotectin, CRP and homocysteine plasma or serum levels. The subjects were either self- or physician-referred asymptomatic individuals and had had an EBCT scan within the previous 2 years (usually within the previous 6 months) to testing of their plasma or serum for the concentration of calprotectin, CRP and homocysteine.

The results of an odds ratio analysis carried out on the co-variance data obtained from the study are shown in Table 1.

Table 1

Risk markers (N1#pos, N2#neg)	**Odds ratio**		
	Calcium	**Calprotectin**	**CRP**
Calcium Score (co 100) N1=100, N1=100	-	-	-
Calprotectin (co 0.7 mg/L) N1=71, N2=121	2.05	-	-
CRP (co 1.69 mg/L) N1=72, N2=128	1.0	2.6	-
Hcy (co 12 μmol/L) N1=11, N2=189	1.21	1.3	1.52
(co 10 μmol/L) N1=42, N2=158	1.63	1.72	1.12

co = cut off

A high odds ratio indicates a high degree of co-variation between the tests. It can be seen from Table 1 that the test for calprotectin gives the highest odds ratio to calcium score and therefore it can be deduced that calprotectin has the highest degree of covariance with calcium score out of calprotectin, CRP and homocysteine.

Additionally, calprotectin gives a high odds ratio with CRP, another marker for CVD.

Chi-Squared Analysis using Minitab

A chi-squared test on the above data, using the same cut-offs as the odds-ratio test, showed significant (P 0.042) agreement between the calcium score and calprotectin results. In contrast, neither the test for CRP nor the test for homocysteine showed any significant co-variance with calcium score.

Agreement Rate Against Calcium Score

The accuracy of each of the calprotectin, CRP and homocysteine (Hcy) tests at the cut-off levels in Table 1 as a test for potential to CVD was also assessed assuming that a calcium score >100 is reflective of high risk to CVD.

The results of the analysis are shown in Table 2.

Table 2

Calprotectin & CRP incorrect	Calprotectin correct & CRP incorrect	Calprotectin incorrect & CRP correct	Calprotectin & CRP correct
55 27.5%	45 22.5%	28 14%	72 36%
Calprotectin & Hcy incorrect	**Calprotectin correct & Hcy incorrect**	**Calprotectin incorrect & Hcy correct**	**Calprotectin & Hcy correct**
48 24%	44 22%	35 17.5%	73 36.5%

Agreement rate against calcium for calprotectin = 58.5% (45+72/200)

Agreement rate against calcium for CRP = 50% (28+72/200)

Agreement rate against calcium for homocysteine = 54% (35+73/200)

Mann-Whitney Test using Minitab

A Mann-Whitney analysis to test for significant increases in the medians of each of calprotectin, CRP, and homocysteine concentration in the high (CS > 100) and low (CS < 100) calcium score groups was also carried out on the above data.

The results showed that the median values for calprotectin and homocysteine concentration were significantly raised in the high calcium group (P = 0.0112 and 0.0059 respectively) whereas CRP did not show any significant difference in either group.

Claims

1. An assay method for the detection of potential for CVD or propensity to CVD in a human or non-human animal subject, said method comprising assessing the concentration of calprotectin in a calprotectin-containing sample taken from said subject.

2. An assay kit for use in the method of the invention, said kit comprising reagents and instructions for the performance of the assay method and for the interpretation of the results and, optionally, calprotectin-containing reference samples, and optionally, a detector.

3. A method for the determination of calprotectin in a calprotectin-containing body fluid, said method comprising the steps of:

(a) obtaining a calprotectin-containing liquid sample of or derived from said fluid;

(b) contacting said sample of said body fluid with an, optionally nanoparticle-bound, anti-calprotectin antibody or antibody fragment, to bind said calprotectin;

(c) optionally, adding an opacity enhancer; and

(d) assessing the calprotectin content by turbidimetry.

4. A diagnostic turbidimetric assay according to the invention, said kit comprising:

preferably, a calprotectin solution of known concentration and more preferably a set of such solutions having a range of calprotectin concentrations;

one or more anti-calprotectin antibodies or antibody fragments, optionally immobilised on a solid support (e.g. nanoparticles);

preferably, a light transmitting vessel;

preferably, an opacification enhancer; and preferably, a detector.

5. An automated apparatus to receive a calprotectin-containing body fluid sample, apply the anti-calprotectin antibody or antibody fragment, optionally immobilised on a solid support (e.g. nanoparticles), optionally apply an opacification enhancer, and determine calprotectin content.